Hantavirus Disease Outbreak in Germany: Limitations of Routine Serological Diagnostics and Clustering of Virus Sequences of Human and Rodent Origin

Stefan Schilling,1,† Petra Emmerich,1 Boris Klempa,2 Brita Auste,2 Ebbo Schnaith,3 Herbert Schmitz,1 Detlev H. Krüger,2,* Stephan Günther,1, and Helga Meisel2

Department of Virology, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg,1 Institute of Virology, Helmut-Ruska-Haus, Charité University Hospital, Berlin,2 and Laboratory Schubach and Associates, Passau,3 Germany

Received 22 December 2006/Returned for modification 7 March 2007/Accepted 1 July 2007

In Europe, hemorrhagic fever with renal syndrome results mainly from infection with Puumala virus (PUUV) or Dobrava virus. For 31 patients from a hantavirus disease outbreak in Lower Bavaria, a district in southeast Germany, serodiagnosis was undertaken by enzyme-linked immunosorbent assay, immunofluorescence assay, and immunoblot analysis. In a few of these cases, however, PUUV-specific typing of antibodies by these standard assays failed and a virus neutralization assay under biosafety level 3 conditions was required to verify the infection by this virus type. PUUV RNA was amplified by reverse transcription-PCR from acute-phase sera of three patients and was found to be very closely related to virus sequences obtained from bank voles (Clethrionomys glareolus) trapped in the same area. These findings link the outbreak with a novel PUUV lineage, “Bavaria,” circulating in the local rodent population. The Bavaria lineage associated with the outbreak is only distantly related to other PUUV lineages from Germany.

Two human zoonoses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (also named hantavirus pulmonary syndrome) are caused by infections with hantaviruses. Case fatality rates can reach up to 50%. The agents, negative-strand RNA viruses forming the genus Hantavirus within the Bunyaviridae family, are transmitted to humans from their respective rodent reservoirs (8, 13, 19). In Central Europe, HFRS occurs mainly due to infections by Puumala virus (PUUV) transmitted from the red bank vole (Clethrionomys glareolus) and by a Dobrava virus (DOBV) lineage, named DOBV-Aa, carried by the striped field mouse (Apodemus agrarius) (7, 8, 10, 23). Infections by both PUUV and DOBV-Aa usually cause mild to moderate HFRS, which is also known as nephropathia epidemica (22). Germany reports up to 450 HFRS cases per year (18).

Since viremia in HFRS patients is short termed and characterized by fluctuating RNA levels, molecular genetic approaches are not common in the identification of human infections. Instead, laboratory diagnosis is based on serology. Usual methods are enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) formats based on the immunodominant nucleocapsid protein (N) antigen and virus-infected cells, respectively, to detect antibodies of IgM class and IgG classes. Also, an immunoblot assay, based on the use of N antigen, has been established. The majority of hantavirus-infected patients present detectable IgM class antibodies already at the onset of disease. In only few cases was the IgM response found to be delayed. IgM disappears within months; however, when using sensitive test formats, it can be detected in single cases as late as 2 years after the acute phase of infection (8, 12). The IgG response is long lasting but sometimes delayed; in about 10% of acute-phase, IgM-positive sera, no IgG can be found with routine assays (8, 20).

Here we describe the serological and molecular epidemiological investigation of an outbreak of hantavirus disease in Lower Bavaria, a district in southeast Germany (Federal State of Bavaria), in 2004. We tested early and, in some cases, follow-up sera of 31 patients, showing that the outbreak was caused by Puumala virus. In certain cases, ELISA, IFA, or immunoblot assays were unable to support the diagnosis of PUUV infection because of extended cross-reaction of IgM and even IgG antibodies to other hantavirus antigens. In these cases, focus reduction neutralization testing (FRNT) confirmed PUUV infection. PUUV sequences were amplified from sera from three patients and compared with those from bank voles trapped in the same area where those three patients resided. The human infections were caused by a novel genetic lineage of the PUUV species, called Bavaria.

MATERIALS AND METHODS

Patients and sera. Serum samples were collected by local physicians from patients with clinical signs of HFRS (nephropathia epidemica) and sent to the German Reference Centers for Tropical Infections (Hamburg) and Hantavirus Infections (Berlin) for diagnosis. First serum samples were taken during the acute phase (n = 31). In some cases, a second sample (n = 13) was collected during convalescence.

Rodent trapping. Rodents were trapped at Bavarian National Park (village of Neuschönau) located in the vicinity of the sites where human infections occurred. Pleural and abdominal fluids, lungs, spleens, kidneys, and urinal bladders were stored at −70°C.

Serological assays. All human serum samples and body fluids of rodents were tested for IgG and IgM antibodies to PUUV, DOBV, or Hantaan virus (HTNV).
by IFA and ELISA. IgG and IgM IFA were performed with HTNV (strain A549/76-118) and PUUV (strain B1NE 568/8)-infected Vero E6 cells. Cells were spread onto slides, air dried, and acetone fixed. Samples were serially diluted in twofold steps in phosphate-buffered saline starting with an initial dilution of 1:10, added to the cells, and incubated for 90 min at 37°C. After washing in phosphate-buffered saline, slides were incubated with fluorescein isothiocyanate-labeled rabbit anti-human IgG and IgM antibodies (which are gamma-chain and mu-chain specific, respectively; SIFIN, Berlin, Germany) at 37°C for 25 min. For rodent samples, fluorescein isothiocyanate-labeled goat anti-mouse IgG/IgM (SIFIN, Berlin, Germany) was used. IgG titers of 1:40 or more and IgM titers of 1:20 or more were considered positive.

IgG and IgM ELISA was performed using recombinant nucleocapsid protein of PUUV, DOBV, and HTNV as described previously (12). Titers of 1:400 or more were considered positive. Selected sera were tested using commercially available Western blots (recomBlot; Mikrogen, Martinsried, Germany) according to the manufacturer’s instructions and by FRNT. FRNT was performed with PUUV, DOBV, and HTNV under biosafety level 3 conditions as described previously (5).

Reverse transcription-PCR (RT-PCR), cloning, and sequencing. For the isolation of RNA from patient sera, the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) was used. Serum (140 μl) was added to 560 μl of buffer AVL with carrier RNA, and the standard QIAamp viral RNA mini spin protocol was performed. RNA was eluted to 60 μl of buffer AVE. Total cellular RNA of seropositive rodents was extracted from homogenized lung and kidney tissues by using TRIzol reagent (GibcoBRL, Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol. RNA was reverse transcribed with murine leukemia reverse transcriptase (Invitrogen, Karlsruhe, Germany) by using random hexamers. PUUV-specific nested PCR was performed as described previously (1) using the TEMPase hot-start master mix kit (BIOMOL, Hamburg, Germany). In addition, overlapping fragments were generated by two seminested PCRs to obtain the complete nucleotide sequence of the first PCR product.

The amplified products were cloned into pCR 2.1 TOPO vector (TA cloning kit; Invitrogen, Leek, Netherlands). At least three recombinant plasmids were sequenced in both directions, and the consensus sequence was determined. Dideoxy sequencing was performed on a LICOR sequencer using the AutoRead kit (Pharmacia-Biotech, Freiburg, Germany) as described by the manufacturer.

Sequence and phylogenetic analyses. The sequence data were further analyzed using the BioEdit software package (4). Sequences were aligned using the CLUSTALW algorithm (25). The reliability of the alignment was checked using DotPlot analysis implemented in the BioEdit software package. The alignment was tested for phylogenetic information by likelihood mapping analysis (24) implemented in the TREE-PUZZLE 5.2 software program (21). A neighbor-joining phylogenetic tree was constructed using the PAUP* software program. The Tamura-Nei evolutionary model (TN93) was used to calculate genetic distances. Bootstrap statistical support values were calculated on 10,000 bootstrap replicates.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in GenBank under accession numbers EU004029 to EU004036.

RESULTS

Epidemiology of the outbreak. The region around Passau in Lower Bavaria was not a hot spot of hantavirus transmission in Germany before 2004. In the previous years, no cases to three cases per annum were reported from the same district (17). In contrast, between January and November 2004, we confirmed acute hantavirus infection in 31 patients (mean age, 36 years; 78% male) by laboratory investigation. Until May 2004, four sporadic infections were confirmed acute hantavirus infection in 31 patients (mean age, 36 years; 78% male) by laboratory investigation. Until May 2004, four sporadic infections occurred. The peak of the epidemic was observed between June and October with 27 cases. The epidemic curve suddenly declined in November (Fig. 1). Most of the cases occurred in a radius of about 25 km in a region of the Bavarian Forest north of the city of Passau (Fig. 2).
TABLE 1. Serological testing of samples from HFRS patients using different assays

<table>
<thead>
<tr>
<th>Virus antigena and serological assay</th>
<th>Acute phase (n = 31)</th>
<th>Convalescent phase (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Positive</td>
<td>Reciprocal GMTb</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>------------</td>
<td>----------------</td>
</tr>
<tr>
<td>PUUV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM ELISA</td>
<td>100.0</td>
<td>1,796</td>
</tr>
<tr>
<td>IgM IFA</td>
<td>93.8</td>
<td>107</td>
</tr>
<tr>
<td>IgG ELISA</td>
<td>93.8</td>
<td>5,489</td>
</tr>
<tr>
<td>IgG IFA</td>
<td>100.0</td>
<td>2,772</td>
</tr>
<tr>
<td>HTNV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM ELISA</td>
<td>43.8</td>
<td>635</td>
</tr>
<tr>
<td>IgM IFA</td>
<td>87.5</td>
<td>40</td>
</tr>
<tr>
<td>IgG ELISA</td>
<td>56.3</td>
<td>491</td>
</tr>
<tr>
<td>IgG IFA</td>
<td>96.9</td>
<td>162</td>
</tr>
<tr>
<td>DOBV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM ELISA</td>
<td>62.5</td>
<td>481</td>
</tr>
<tr>
<td>IgG ELISA</td>
<td>81.3</td>
<td>1,204</td>
</tr>
</tbody>
</table>

a N antigen for ELISA; virus-infected cells for IFA.
b GMT of positive samples.

All patients met the clinical criteria of nephropathia epidemica (8), and most of them required hospitalization due to impaired renal function. Neither severe hemorrhagic complications nor fatal courses of infection were observed. Impaired renal function. Neither severe hemorrhagic complications nor fatal courses of infection were observed.

Since southeast Germany is a geographical region where Clethrionomys glareolus, but not Apodemus agrarius, is prevalent as a hantavirus reservoir, PUUV could be expected as the etiological agent of the outbreak.

Serological investigation of patient sera. Serum samples from the acute phase of illness were available from 31 patients. A follow-up serum sample was collected 1 to 20 weeks later from 13 of the 31 patients. All 44 sera were tested for IgM and IgG antibodies by IgM and IgG ELISA, respectively, on the basis of PUUV, DOBV, and HTNV nucleocapsid protein antigens and by IFA using PUUV- or HTNV-infected cells. Because HTNV is antigenetically closely related to DOBV (8) and HTNV-infected cells were immediately available, they were used to detect DOBV-specific antibodies by IFA.

The largest number of positive samples and the highest reciprocal geometric mean titer (GMT) of positive samples was seen in the PUUV-specific assays, suggesting that the outbreak was caused by PUUV (Table 1). All acute-phase sera were IgM positive by PUUV-specific ELISA. The percentage of IgM-positive samples and their GMTs were lower by IFA than by ELISA. Similar differences were observed for the follow-up samples, suggesting that IFA is less sensitive than ELISA for IgM detection. No major differences were seen between the two methods with respect to IgG detection.

However, in a few cases, IgM titers against heterologous DOBV and HTNV antigens were similar (by IFA) or even higher (by ELISA) (Table 2). Antibody specificity in these sera was further tested by immunoblot analysis. In all cases but one, higher IgM reactivity against PUUV was demonstrated (Table 2).

Table 3 summarizes results for four sera with indeterminate or DOBV-/HTNV-specific IgG typing by ELISA. All sera reacted with the highest titers against PUUV by IFA, whereas immunoblot analysis identified dominant PUUV reactivity only in serum sample 361. Immunoblot results for serum samples 2395 and 2813 corresponded to the indeterminate typing results by ELISA. In the case of serum sample 2395, the blot analysis of a consecutive serum sample (sample 2753) from the same patient showed a slight PUUV antigen preference.

Due to the particularly conflicting IgG results for serum samples 361 and 2813 by ELISA, immunoblot analysis, and IFA, we decided to use FRNT to determine the specificity of the sera. The typing of neutralizing antibodies by FRNT is the method of choice to determine the hantavirus species that led to the infection of a patient (8). Table 4 demonstrates that serum samples 361 and 2813 neutralized PUUV clearly better than they neutralized DOBV or HTNV, strongly suggesting that the corresponding patients had been infected by PUUV.

Testing of patient sera by PUUV RT-PCR. RNA was extracted from all sera and tested by PUUV S segment-specific RT-PCR. Genetic material was amplified from only three sera taken early after the onset of disease. The three patients (designated H139, H151, and H201) were living in different villages in the Bavarian Forest north of the city of Passau (Fig. 2). All three cases were characterized by acute onset with fever, myalgia, back pain, and abnormal fatigue. Acute renal failure was accompanied by thrombocytopenia and an increase in serum creatinine. Hemodialysis treatment was not necessary, and all three patients recovered. Interestingly, in one of the three patients (H151), blurred vision, conjunctival petechiae, and lung hemorrhage were also observed.

TABLE 2. Exceptional results of IgM assays without clear PUUV preferencea

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>ELISA</th>
<th>IFA</th>
<th>Immunoblot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PUUV</td>
<td>DOBV</td>
<td>HTNV</td>
</tr>
<tr>
<td>235</td>
<td>400</td>
<td>1,600</td>
<td>6,400</td>
</tr>
<tr>
<td>236</td>
<td></td>
<td>1,600</td>
<td>1,600</td>
</tr>
<tr>
<td>2395</td>
<td>6,400</td>
<td>1,600</td>
<td>0</td>
</tr>
<tr>
<td>3126</td>
<td>25,600</td>
<td>1,600</td>
<td>25,600</td>
</tr>
<tr>
<td>3156</td>
<td>1,600</td>
<td>1,600</td>
<td>1,600</td>
</tr>
<tr>
<td>3300c</td>
<td>1,600</td>
<td>1,600</td>
<td>400</td>
</tr>
</tbody>
</table>

a ELISA, IFA, or blot data not directly supporting the diagnosis of PUUV infection are shown in bold. −, no reaction; +, same intensity as the cutoff band; ++, strong intensity (stronger than the cutoff band); SEOV, Seoul virus. Numbers are reciprocal titers.
b RT-PCR data available.
c Sampled from the same patient as that for serum sample 3156 but obtained 2 weeks later.
infiltration were observed during the first days of disease. The sequences of the PCR products revealed infection by a specific PUUV strain (see below), confirming the serological findings and providing direct evidence that PUUV caused the epidemic.

**Rodent trapping and analysis.** To investigate whether the PUUV strain identified in the patients was also prevalent in the local rodent population, 38 bank voles were trapped in November 2004 in the outbreak region, National Park “Bavarian Forest” (Fig. 2). Seven of 38 animals (18.4%) were found seropositive for PUUV by IFA. Eight animals, including two seronegative voles, were positive by PUUV S segment RT-PCR analysis of lung and kidney tissues. The amplified PCR products from the rodents were sequenced and used for molecular epidemiological analysis.

Furthermore, we wondered whether the PUUV strain is specific to the Bavarian region. Therefore, rodents trapped in other regions of Germany were included in the study. Three of 49 bank voles (6.2%) trapped in August 2004 in northeast Germany near the city of Lübeck and five of 16 bank voles (31.25%) trapped in June 2005 in West Germany near the city of Koblenz tested positive by PUUV-specific RT-PCR. The amplified DNA fragments were sequenced and included in the phylogenetic analysis.

**Sequence and phylogenetic analyses.** Partial S segment sequences (616 nucleotides, positions 354 to 969) of PUUV from sera of the three patients (designated strains H139/04, H151/04, and H201/04), kidney tissues of three bank voles trapped in the outbreak region (designated strains ClgM26/04, ClgM26/04, and ClgM37/04), and kidney tissues of two bank voles trapped outside the outbreak region (designated strains D208/04 [from the Lübeck region in North Germany] and Klbl3/05 [from the Koblenz region in West Germany]) (Fig. 2) were subjected to sequence comparisons and phylogenetic analysis.

The sequence analysis showed that all human and rodent sequences from the outbreak region were very closely related, with 97.4 to 99.0% sequence identity. Altogether, there were 32 polymorphic sites within the alignment of the Bavarian sequences. Only one substitution represents a nonsynonymous mutation leading to the conservative amino acid exchange I251M in the putative N protein, where the strains H139/04 and ClgM26/04 encode isoleucine (I) but the other Bavarian strains carry methionine (M) (data not shown). Sequence similarity of the Bavarian strains to other German and European PUUV sequences was rather low (81.4 to 87.0%). The only German PUUV (partial) nucleotide sequence recovered from a patient with HFRS so far (15) showed only 84.7 to 85.3% similarity to the nucleotide sequence of the Berkel strain (accession no. L36943). Interestingly, the most related sequence is not from a German strain but from strain Opina-916 (accession no. AF294652) obtained from a bank vole captured in Slovakia (9).

Phylogenetic analysis (neighbor joining) of 60 PUUV sequences confirmed the clear molecular epidemiological link between PUUV strains from patients and rodents collected in the outbreak area. All Bavarian sequences formed a well-supported monophyletic group, indicating that the outbreak had been caused by a specific Bavarian lineage of PUUV that differs from lineages found in other parts of Germany. Even a direct coupling of patient- and rodent-associated sequences could be observed in the cases of H139/04 and ClgM26/04 sequences (Fig. 3).

As expected, all analyzed German sequences clustered within the Central European clade formed by strains from Belgium, Slovakia, and Germany. Within this clade, at least four lineages can be distinguished further. The strains from Bavaria form one of them. The strain from Slovakia represents the second lineage. The third group is formed by strains from Belgium and northwest Germany (Cg-Erft, accession no. AJ238779) and by our strains Klbl3/05 from west Germany and D208/04 from north Germany. The fourth lineage is represented by another sequence from northwest Germany (Berkel, accession no. L36943) obtained from a patient with severe HFRS (15). Interestingly, the statistical support for the monophyletic group of the three above-defined lineages (bootstrap value of 88 [data not shown]) was removed by the addition of the latter sequence into the data set.

**DISCUSSION**

Here we report the serological and molecular epidemiological investigation of an outbreak of hantavirus disease involving 31 patients in southeast Germany. The patients exhibited clinical symptoms and biochemical findings typical of HFRS cases...
occurring in Germany. The findings show that the outbreak was caused by a novel strain of PUUV that was only distantly related to other German PUUV strains.

In most cases, serological testing identified PUUV as the causative agent. In contrast to the common view, standard methods such as ELISA, IFA, and immunoblot analysis were not always able to determine the causative hantavirus species. In these cases, testing of consecutive sera or typing of neutralizing antibodies by FRNT was needed to confirm the virus-specific diagnosis of PUUV infection. Our findings demonstrate that due to the cross-reactivity of sera, serotyping for diagnoses of individual patients as well as seroepidemiological studies may sometimes not correctly identify the causative hantavirus if typing is based only on ELISA, IFA, or immunoblot analysis.

Besides the neutralization assay, RT-PCR and sequencing provide clear information on the causative virus species. However, it is known that PUUV infection leads to a short and fluctuating viremia. This is why the recovery of viral nucleic acid sequences from patient sera is possible during only the first days after the onset of disease and may even fail during this time. We were able to amplify viral sequences from early sera of three patients. Sequence analysis confirmed the diagnosis of PUUV infection and indicated that the outbreak was caused by a novel virus strain.

The trapping of bank voles in the vicinity of human cases...
allowed us to compare nucleotide sequences of human and rodent origin from the hantavirus disease outbreak. Virus sequences from patients were phylogenetically closely related to those of the bank voles, suggesting that the patients had been infected by the prevalent PUUV strains circulating in this area. This view is supported by an independent study, which was focused on the reservoir species in the outbreak area (3). All sequences obtained from these animals were also closely related to the human sequences determined in our study (up to 100% identity).

In the analyzed S segment region, we found a degree of informative nucleotide substitutions of 2.1% between the Bavarian sequences of human and rodent origin accompanied by one amino acid difference. In general, we and others (6, 16) did not see specific mutations that can be associated with the adaptation of a rodent-derived hantavirus to a human host. Probably, such a mutation(s) is not necessary as long as the infected patient is a "dead-end host" for the virus and a complete adaptation to the new host with high viraemia, enabling its human-to-human transmission, does not occur. There are only a few reports about interhuman transmission of Andes hantavirus; however, no comparison between viral sequences from these persons and their rodent sources of infection has been undertaken (11, 14).

In addition to the PUUV strains from the outbreak in Bavaria, we included novel PUUV sequences from North and West Germany in the phylogenetic analysis in order to determine the evolutionary origin of the outbreak strain in more detail and to expand our view about the molecular diversity of PUUV circulating in Central Europe (Fig. 3). To our surprise, the Bavarian lineage did not cluster with the German strains from Koblenz and Lübeck but was equidistant from the Slovakian PUUV on the one hand and the German and Belgian strains on the other hand. Thus, the phylogenetic reconstruction suggests the existence of at least two well-supported Central European lineages of PUUV (Bavaria; Belgium/NW Germany). The strains AF294652 (Slovakia) and L36943 (NW Germany) may occupy separate lineages but also may join one of the supported lineages; the exact phylogenetic relationship between these lineages could not yet be resolved on the basis of the existing sequence information. Strains from another Central European country, Austria (1), belong to a different phylogenetic clade, together with southeast European strains from the Balkans (Fig. 3).

The reason for the outbreak of hantavirus disease in the Bavarian Forest in 2004 is not known. The local accumulation of cases is remarkable since, in Germany, the number of recorded HFRS cases had not increased compared with the annual average. One might speculate that 2004 was "high mast year" for Clethrionomys glareolus animals in this wooded area, although underlying ecological changes are unknown. A higher rodent density facilitates the spreading of virus between animals and its transmission to humans. Similar reasons were assumed to explain the first outbreak of hantavirus cardiopulmonary syndrome in the southwestern United States in 1993 (2). In addition, information in the local press in the Bavarian Forest area at the beginning of the hantavirus outbreak could have increased the awareness of physicians of this disease and might have contributed to the high number of recorded HFRS cases.

ACKNOWLEDGMENTS

We thank M. Kramski, H. Lerch, and M. Pohl (Berlin) for help in the laboratory investigations as well as G. Haider (Grafenau), W. Hautmann (Oberschleissheim), K. Zimmer (Koblenz), and many other colleagues for their support of our study. Experimental work in Berlin was supported by Deutsche For- schungsgemeinschaft (grant KR 1293/2-4).

REFERENCES

